

Technological Advances in the Molecular Biology of *Leptospira*

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Abstract

Pathogenic members of the genus *Leptospira* have been refractory to genetic study due to lack of known mechanisms of genetic exchange. To bypass this limitation, several techniques have been useful for *Leptospira* gene discovery, including heterologous complementation of *Escherichia coli* mutants, screening of DNA libraries with probes, and random sequence analysis. Construction of combined physical and genetic maps revealed the presence of two circular chromosomal replicons. The organization of the *L. interrogans* genome is quite variable, with genetically similar strains differentiated by many rearrangements. These rearrangements likely occur through recombination between repetitive DNA elements found scattered throughout the genome. Analysis of intervening sequences and genes encoding LPS biosynthetic enzymes provide evidence of lateral transfer of DNA between *Leptospira* spp. We have also gained insight into the biology of these bacteria by analyzing genes encoding LPS and outer membrane proteins (OMPs). Some of these OMPs are differentially expressed. Characterization of mechanisms governing the expression of the OMP genes should provide insight into host-parasite interactions. Furthermore, recent advances in heterologous expression of leptospiral OMP genes are opening new avenues of vaccine development.

Introduction

Leptospira is a genetically diverse genus containing several species: *L. alexanderi*, *L. biflexa*, *L. borgpetersenii*, *L. inadia*, *L. interrogans*, *L. kirschneri*, *L. meyeri*, *L. noguchii*, *L. parva*, *L. santarosai*, *L. weilii*, *L. wolbachii*, and several

unnamed genomospecies (Brenner *et al.*, 1999). These bacteria are antigenically diverse. Changes in the antigenic composition of lipopolysaccharide (LPS) are thought to account for this antigenic diversity. The presence of more than 200 recognized antigenic types (termed serovars) of pathogenic leptospires have complicated our understanding of this genus. In several cases, members of different species are serologically indistinguishable and belong to the same serovar. For example, strains of serovar hardjo have been isolated which belong to the species *L. interrogans*, *L. borgpetersenii*, and *L. meyeri* (Brenner *et al.*, 1999).

Leptospirosis, caused by the pathogenic leptospires, is one of the most widespread zoonotic diseases known. In spite of significant genetic diversity between species (Brenner *et al.*, 1999) these bacteria cause clinically similar diseases. Two forms of leptospirosis are recognized: an acute, life-threatening infection; also a chronic disease, with little outward signs of infection. Both forms of the disease involve a systemic infection, resulting from contact with contaminated body fluids. These bacteria concentrate in the proximal convoluted tubules of the kidney. Thus, urine of infected animals is a primary source for new infections. Significant concentrations of bacteria ($>10^6$ cells/ml) have been detected in animal urine, and humans are often infected as the result of environmental exposure involving contact with urine-contaminated water. It has not been shown that pathogenic leptospires replicate outside mammalian hosts in nature. However, the ease with which this disease is transmitted through contaminated water suggests that these bacteria adapt well and must have substantial genetic capacity to survive in such diverse environments.

Lack of known mechanisms for genetic exchange between leptospires has impeded progress in the genetic analysis of these bacteria. Three bacteriophages, LE1, LE2, and LE3, have been characterized (Saint Girons *et al.*, 1990). These phage are limited to growth in the saprophyte *L. biflexa* serovar patoc, and appear to undergo a lytic cycle but not a lysogenic cycle (Saint Girons *et al.*, 1990). Transduction has not been detected. Thus, use of phage for classical genetic analysis of *Leptospira* is limited. Recent developments describing construction of a shuttle vector using phage sequences are presented in this issue (Saint Girons *et al.*, 2000). These studies offer hope that genetic manipulation of these bacteria will soon be possible. There is no evidence for conjugation, and a single report of transformation of *Leptospira* (van Riel, 1964) has not been successfully repeated. Despite conflicting reports, it does not appear that *Leptospira* has accessory genetic replicons (linear or circular plasmids). Given these limitations, much of the research on the molecular biology of *Leptospira* has used molecular cloning and heterologous expression of genes in suitable bacterial hosts.

We review here recent progress in characterizing the genetics and cell wall structures of *Leptospira* spp. Attention is given to both the findings, and in several cases, the pitfalls of different techniques used in analysis.

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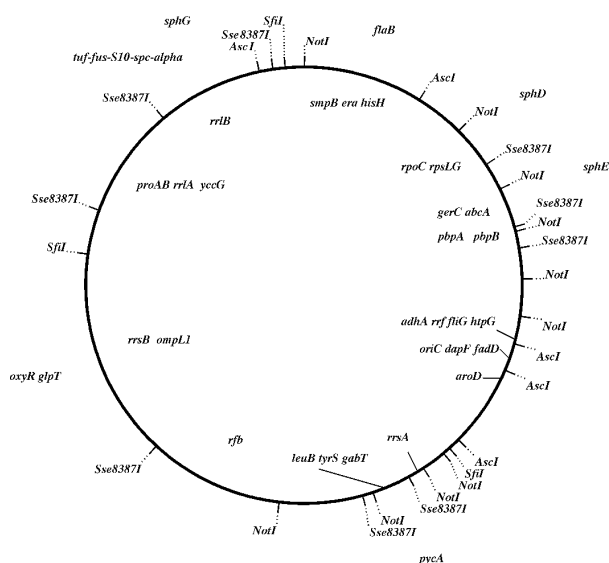


Figure 1. Physical and genetic map of *L. interrogans* serovar pomona. The CI replicon is shown. The position of restriction sites and genes distributed around this replicon are indicated. Newly localized genes are shown on the outside of the map. These genes include: *flaB* (encoding core flagella protein); the *fus-tuf-S10-spc- α* operon; *glpT* (encoding glycerol-3-phosphate transporter); *hepA* (encoding RNA helicase); *oxyR* (encoding oxidoreductase); *pycA* (encoding pyruvate carboxylase); and sphingomyelinase homologs *sphD*, *sphE*, and *sphG*.

Gene Identification

Initial studies for isolation and characterization of *Leptospira* genes used heterologous complementation of *E. coli* amino acid auxotrophs. Physiological studies suggest that *Leptospira* can synthesize most amino acids (Johnson, 1977), and must therefore have a large repertoire of genes encoding these biosynthetic functions. Complementation studies have yielded sporadic success, with isolation of several genes including *asd*, *aroD*, and *dapD* (Baril *et al.*, 1992), *leuB* (Richaud *et al.*, 1990; Ding and Yelton, 1993), *proAB* (Richaud *et al.*, 1990), and *trpEG* (Yelton and Charon, 1984). The *L. meyeri* *metX* and *metY* genes were also isolated by complementation in *E. coli* (Bourhy *et al.*, 1997). However, subsequent enzymatic studies showed that leptospires use a different pathway for methionine biosynthesis than *E. coli* (Belfaiza *et al.*, 1998). Confusing results were obtained during isolation of an *L. biflexa* gene that complemented an *argE* mutation (Zuerner and Charon, 1988). The *argE* complementing activity was associated with a gene homologous to *rpoC*, encoding the RNA polymerase β' subunit. Although this gene can complement the deacetylase-acetyltransferase activity lacking in *E. coli*, it is not clear if this gene functions in arginine biosynthesis in *L. biflexa* (Zuerner and Charon, 1988). These findings underscore the need for a genetic system to validate complementation findings, and that enzymatic analysis should be used to support and clarify complementation results.

The *L. biflexa* *recA* gene was also isolated by complementation of an *E. coli* mutant (Stamm *et al.*, 1991). A surprising finding of those studies was that the cloned *L. biflexa* gene both complemented the DNA repair function of *recA* and could induce prophage λ . These data imply that the *L. biflexa* RecA protein can digest the *cl* repressor

protein. These findings suggest other *Leptospira* DNA repair genes may be isolated by complementation.

Using a probe to the *rfbBA* genes of *Salmonella enterica*, Mitchison *et al.* (1997) cloned an *L. interrogans* serovar copenhageni locus containing *rfbCDBA*. These genes encode enzymes associated with the synthesis of activated rhamnose, a component of LPS. The cloned *rfbB* gene complemented a *rfbB::Tn5* mutant of *S. flexneri*, restoring LPS biosynthesis. Several *L. borgpetersenii* serovar hardjo LPS genes have also been shown experimentally (Kalambaheti *et al.*, 1999). Orf8 encodes the GalE enzyme required for the synthesis of galactose as evidenced by the detection of GalE activity expressed by the cloned orf8 in a *galE* *Salmonella* mutant. Orf13 complemented a *rfbW* mutant of *Vibrio cholerae* demonstrating galactosyl transferase activity (Bulach *et al.*, 2000). A *Pseudomonas aeruginosa* *wbpM* mutant was complemented by Orf10, and thus is involved in fucosamine biosynthesis (Bulach *et al.*, 2000). These experiments are particularly difficult as the biosynthesis of LPS involves several discrete steps, and complementation may be restricted at the levels of substrate specificity or enzyme interactions. The structural composition of *L. interrogans* LPS is unknown, yet successful heterologous complementation of LPS mutants illustrate conservation of the enzymatic steps associated with assembly of these molecules.

Several genes were identified through random sequence analysis studies (Boursaux-Eude *et al.*, 1998). Included among these were several amino acid biosynthetic genes, which have not yet been tested for their ability to complement mutations in *E. coli*. Some interesting genes identified include *gerC*, a gene of unknown function initially associated with spore germination in *Bacillus* spp. The *yccG* gene, encoding methyl glyoxal synthesis, was also cloned. This gene may be associated with the transition from stationary to log-phase growth. Determination of the conditions under which these genes are transcribed may provide insight into the adaptive changes leptospires undergo when entering new environments. Analysis of these genes may prove useful as we seek to characterize regulatory mechanisms governing variable membrane protein expression (see below).

Physical and Genetic Organization

Pulsed-field gel electrophoretic analysis revealed the *L. interrogans* genome is 4.75 to 4.9 Mb in size and has two circular chromosomal replicons: CI, 4.4 to 4.55 Mb, (Figure 1) and CII, 350 Kb (Baril *et al.*, 1992; Takahashi *et al.*, 1998; Zuerner, 1991; Zuerner *et al.*, 1993b). The CI replicon resembles a "typical" bacterial chromosome, containing the rRNA genes and most biosynthetic loci identified to date. One unique feature of this replicon is the dispersal of rRNA genes. In most bacteria, the *rrs*, *rrl*, and *rrf* genes (encoding the 16S, 23S, and 5S rRNAs, respectively) are tightly clustered and co-transcribed (Krawiec and Riley, 1990). However, in *L. interrogans* these genes are not closely linked and are present in unequal numbers. Nucleotide sequencing of the *rrf* locus identified the presence of *jag*, a gene typically found near chromosomal replication origins (Takahashi *et al.*, 1998). Other mapping studies showed this region of the CI replicon has rearranged, separating other *ori* related genes from *rrf*

(Boursaux-Eude *et al.*, 1998). Figure 1 shows an updated map of the CI replicon showing several newly localized loci including the S10-*spc*- α operon and sphingomyelinase-homologs *sphD*, *sphE*, and *sphG*.

Less is known about the CII replicon because separating it from restriction fragments derived from the CI replicon is often difficult. Attempts to purify it have been unsuccessful. The CII replicon is circular, and does not enter agarose gels when intact (Zuerner, 1991). However, in many DNA samples the CII replicon is partially degraded (linear) and enters agarose gels during electrophoresis, thus enabling visualization (Zuerner, 1991). This replicon is common to all pathogenic serovars, shows little size variation (Hermann *et al.*, 1991), and exists at approximately a 1:1 ratio with the CI replicon (Zuerner, 1991). The CII replicon likely functions as a chromosome, as it contains the sole copy of *asd*, an essential gene encoding aspartate semialdehyde aminotransferase (Zuerner *et al.*, 1993b).

Genetic organization of two genetically similar but serologically distinct serovars of *L. interrogans* were compared, revealing that the leptospiral genome is quite variable. Several rearrangements (insertions, deletions, and inversions), some spanning nearly 30% of the genome, have been identified (Zuerner *et al.*, 1993b; Boursaux-Eude *et al.*, 1998). Yet with this extensive chromosomal fluidity, these bacteria retain virulence. By comparison, when such rearrangements occur in other bacterial genera cells often lose viability. Some chromosomal rearrangements may be associated with antigenic variation (Cacciapuoti *et al.*, 1993). Several repetitive DNA elements have been detected in *Leptospira* and contribute to formation of these rearrangements.

Repetitive DNA

Several repetitive DNA elements have been detected in *Leptospira* including several insertion sequences (IS) and intervening sequences (IVS). In addition, a 4.8 kb repetitive element that contains genes encoding enzymes associated with beta-oxidation of fatty acids was recently identified (Takahashi *et al.*, 1999). Other forms of repetitive DNA are present in high copy number and distributed throughout the genome (Zuerner, 1993). Each of these elements may contribute to the genetic heterogeneity seen among leptospiral strains.

IS elements belonging to the IS3-, IS5-, and IS110-families have been detected in *Leptospira spp.* (Boursaux-Eude *et al.*, 1995; Kalambaheti *et al.*, 1999; Zuerner, 1994). IS elements are mobile genetic elements that usually encode the capacity to catalyze transposition to new sites, and often contribute to the generation of rearrangements (Galas and Chandler, 1993). Comparison of genetic organization in two *L. interrogans* serovars suggest that IS1500, an IS3-like element, may help generate these rearrangements (Boursaux-Eude *et al.*, 1998). IS1533, an IS110-like element in *L. borgpetersenii*, may also affect genetic organization. *L. borgpetersenii* strains with more than 40 copies of IS1533 (about 1% of the total genome) have been identified, and some of these elements may transpose (Zuerner *et al.*, 1993a). Discrete clonal populations of *L. borgpetersenii* serovar hardjo were identified through hybridization analysis with IS1533 probes, and genetic organization among these populations may vary because of rearrangements (Zuerner *et al.*,

1993a). The presence of only a few clonal populations, and the lack of changes among strains maintained under routine cultivation *in vitro* suggests genetic changes involving IS1533 are probably rare.

PCR-based techniques that enable simultaneous detection and serovar identification were developed. These techniques place primers within the "unique" central region of an IS facing outward. Thus, DNA amplification of sequences between closely placed elements occurs (Zuerner and Bolin, 1997; Zuerner *et al.*, 1995). The resulting pattern of amplicons can then be used to identify the serovar directly from clinical samples. These diagnostic techniques are of particular use, because primary isolation of pathogenic *Leptospira* can often take months, and these bacteria are quite difficult to accurately type serologically. One result of these analyses suggests that in many strains, complete or partial copies of IS-like elements likely occur near each other in the genome.

Little data is available regarding the nature of sites into which leptospiral IS elements are found. Recent analysis of the *L. borgpetersenii* serovar hardjo LPS biosynthetic locus revealed that it is flanked by copies of IS1533 (Kalambaheti *et al.*, 1999). All of these LPS biosynthetic genes are shared with *L. interrogans* serovar hardjo (de la Peña Moctezuma *et al.*, 1999). Thus, IS1533 may have played a role in lateral transfer of these genes. Evidence suggests IVSs may also be laterally transferred (Ralph and McClelland, 1994). A better understanding of the role of repetitive DNA elements in genetic organization is needed as we characterize the biology of *Leptospira*.

Transcription Analysis

Transcriptional regulation and promoter sequences in *Leptospira* are poorly defined, yet this information is critical for understanding how these bacteria regulate gene expression. A large ribosomal protein gene cluster was characterized, providing insight into *L. interrogans* gene organization and transcription (Zuerner *et al.*, 2000). This locus has a unique organization, sharing similarity to portions of the *E. coli str.* S10, *spc*, and α -operons. Reverse transcriptase (RT)-PCR analysis was used to define the pattern of transcription throughout this locus. This locus comprises a 17.5 kb operon (Zuerner *et al.*, 2000) and is the first *L. interrogans* operon in which the transcripts have been mapped.

Two σ^{70} -like promoters upstream of *fus*, the first gene in this operon, were identified by primer extension experiments and by functional analysis (Zuerner *et al.*, 2000). It should be noted that preliminary experiments using ³³P-labeled primers produced false results (Zuerner, unpublished), but transcription start sites were clearly mapped with ³²P-labeled primers (Zuerner *et al.*, 2000). The *fus* promoter sequences are different from promoters identified upstream of two heat shock loci (Ballard *et al.*, 1993; Ballard *et al.*, 1998). Few *Leptospira* promoters have been characterized. Thus, a σ^{70} consensus sequence is not yet possible. However, initial studies suggest that *Leptospira* promoters are similar to those of other eubacteria, and function in *E. coli* (Zuerner *et al.*, 2000). Application of RT-PCR analysis to RNA extracted from bacteria obtained from different environments should be useful to understanding the processes by which these bacteria adapt to different growth conditions.

Analysis of Potential Virulence Factors

Acute leptospirosis induces a complex array of clinical signs. One feature often seen during infection is hemolytic anemia. A proteinaceous hemolysin from *L. interrogans* co-purifies with sphingomyelinase, suggesting that the same protein may be responsible for both observed activities (Berhneimer and Bey, 1986). The function of leptospiral sphingomyelinase is unknown, but it is speculated that it could serve dual functions in obtaining both iron and free fatty acids for growth. Several hemolysins have been identified in *Leptospira* spp. An *L. borgpetersenii* serovar hardjo gene, *sphA*, was isolated by its ability to confer hemolytic activity to *E. coli* cells (del Real *et al.*, 1989). Subsequent analysis showed that *sphA* has an open reading frame with the potential to encode a 63 KD protein (Segers *et al.*, 1990). However, in *E. coli* the product of this gene is a 39-KDa protein (del Real *et al.*, 1989). This discrepancy may be due to post-translational processing, or may be associated with differences in the transcriptional or translational signals used during expression in this heterologous host. Despite these discrepancies, cell lysates of *E. coli* harboring *sphA* had sphingomyelinase activity (del Real *et al.*, 1989; Segers *et al.*, 1990), showing that hemolytic and sphingomyelinase activities coincided.

SphA shares significant regions of similarity to the *Staphylococcus aureus* β -hemolysin and the *Bacillus cereus* sphingomyelinase C (Segers *et al.*, 1990). Sequence information is also available for two hemolysins *sphH* and *sphX* (Accession numbers U89708 and AF200703) cloned from *L. borgpetersenii* and *L. interrogans*, respectively. The serovar lai *sphH* gene is next to *lipL32*, the major outer membrane protein (mOMP). The predicted product of *sphH* is similar to *sphA*, but *sphX* appears to encode a hemolytic protein unrelated to the sphingomyelinases. The finding that *sphA* and *sphH* are similar is expected based on a hybridization analysis that showed pathogenic *Leptospira* typically have several discrete *sph*-like sequences (Segers *et al.*, 1992). Three *sph* homologs were localized in *L. interrogans* strain RZ11 (Figure 1) and are designated *sphD*, *sphE*, and *sphG*.

Leptospiral LPS

The studies outlined above help to provide a framework for unraveling the genetics of these bacteria. However, genetics is but one facet of biology, and a primary focus of our laboratories is to develop effective vaccines. To better understand the biology of these bacteria, characterization of the constituents of the outer membrane is essential.

Leptospiral LPS contributes to the pathology associated with disease and comprises the major surface component of leptospires (Figure 2). Studies with monoclonal antibodies have shown that LPS is the target for agglutinating and opsonizing antibodies (Farrelly *et al.*, 1987). Thus, LPS plays a key role in immunity to infection. Indeed, anti-LPS monoclonal antibodies provide passive protection against infection and purified LPS can stimulate active immunity (Jost *et al.*, 1986; Midwinter *et al.*, 1990). As agglutinating antigens, LPS molecules are also important for serological classification of leptospires.

Early reports of antigenic carbohydrate preparations extracted from leptospires used phenol, alkali, acetone or other solvents. Terms such as TM (for type specific main)

antigen, ESS (for erythrocyte sensitizing substance), LLS (for LPS-like substance), or PAg (for protective antigen), have been used for these extracts, but all of them clearly contained LPS or LPS components as major constituents. The structure of LPS remains unknown. However, chemical analysis of leptospiral LPS showed the presence of common hexoses, amino hexoses and pentoses, and some sugars found more rarely as LPS components, such as xylose and arabinose (Vinh *et al.*, 1986; 1989). Methylated and O-acetylated sugars have also been reported (Yanagihara *et al.*, 1983). The detection of KDO in the LPS of *L. interrogans* serovar copenhageni and *L. borgpetersenii* serovar hardjo is consistent with a composition similar to that of typical gram-negative LPS (Vinh *et al.*, 1989).

Despite the structural, biochemical and immunological similarity of leptospiral LPS to gram-negative bacterial LPS, it is at least 10-fold less toxic for animals or cells. Nevertheless, leptospiral LPS can activate macrophages and act as a B-cell mitogen (Isogai *et al.*, 1990a; 1990b). It has been proposed that the reduced toxic activity is a result of the absence of β -hydroxy-myristic acid.

Recent genetic analysis has identified the *rfb* loci involved in the biosynthesis of the LPS O-antigen in several leptospiral serovars. Rhamnose is a component sugar of all serovars analyzed to date. The four genes encoding enzymes required for the biosynthesis of dTDP-rhamnose from glucose were first cloned from *L. interrogans* serovar copenhageni (Mitchison *et al.*, 1997). Genes encoding rhamnosyl transferases were found downstream of the rhamnose biosynthetic cluster.

Subsequent work delineated the *rfb* locus in *L. borgpetersenii* serovar hardjo (Kalambaheti *et al.*, 1999). This locus contains 31 ORFs that encode enzymes involved in the biosynthesis of activated sugars, glycosyl transferases, and sugar processing and transport proteins. Three sugar biosynthetic clusters are involved in the synthesis of rhamnose (orfs24-27), galactose, mannose and fucosamine (orfs6-11) and a dideoxy-amino-hexose (orfs18-20). A putative flippase and O-antigen polymerase were also identified and other glycosyl transferases of unknown specificity. Leptospiral LPS may be assembled via the classical Wzy (Rfc) dependant pathway. Interestingly, the *L. borgpetersenii* serovar hardjo locus is bounded by IS1533 elements, raising the possibility of horizontal acquisition. In addition, an IS5-like element is found between orf14 and orf15.

The *rfb* loci of serologically indistinguishable representatives of serovar hardjo from *L. interrogans* and *L. borgpetersenii* are similar, but lack IS elements (de la Peña-Moctezuma *et al.*, 1999). However, detailed comparison of the sequences revealed that the *L. interrogans* locus consists of four segments. Two of these (orf1-14 and orf21-22) resemble *L. borgpetersenii* serovar hardjo while the orf15-20 and orf23-31 clusters show greater sequence identity to other *L. interrogans* serovars (de la Peña-Moctezuma *et al.*, 1999).

A comparison of the *rfb* loci of ten other *L. interrogans* serovars revealed conservation in the region from orf11 to orf31. Genes upstream of orf11 vary among serovars, or show more genetic drift. This region thus appears to encode biosynthetic enzymes and glycosyl transferases responsible for serovar specificity and some genes of unknown function.

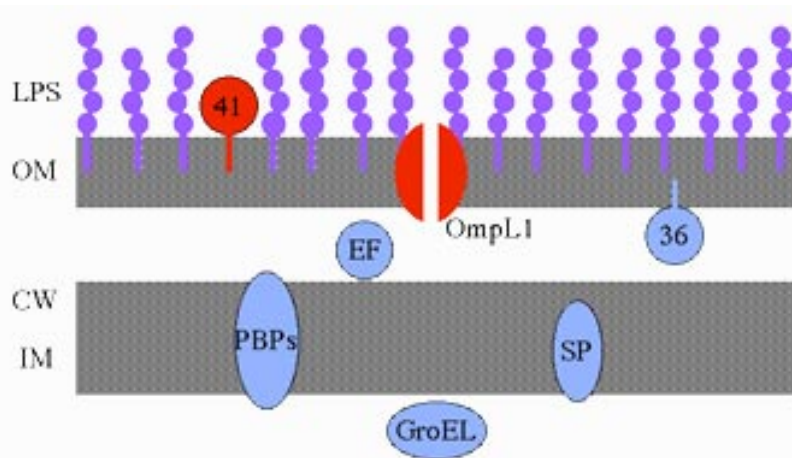


Figure 2. Model of Leptospiral Membrane Architecture. Model of leptospiral membrane architecture showing the location of outer membrane (OM) components lipopolysaccharide (LPS), the porin, OmpL1, and the lipoproteins LipL41 and LipL36. The endoflagella (EF) are located in the periplasm between the outer and inner membranes. The peptidoglycan cell wall (CW) is associated with the inner membrane (IM). Inner membrane proteins include penicillin-binding proteins (PBPs) and signal peptidase (SP). The heat-shock protein (GroEL) is located primarily in the cytoplasm.

Leptospiral Outer Membrane Proteins (OMPs)

An understanding of leptospiral OMPs require an appreciation of the distinctive double-membrane architecture of spirochetes. A model of the cell wall structure is shown in Figure 2. The composition and organization of these membrane layers share characteristics with both Gram-positive and Gram-negative bacteria. As in Gram-positive bacteria, the cytoplasmic membrane of spirochetes is closely associated with the peptidoglycan cell wall. Spirochetes also have an outer membrane that provides a barrier shielding underlying antigens, such as the endoflagella, from the outside environment.

The leptospiral surface is dominated by LPS side chains (Figure 2). The carbohydrate antigens on the leptospiral surface are accessible to antibody, and analysis of leptospiral motility revealed that the outer membrane is quite fluid (Charon *et al.*, 1981). This feature is in contrast to the outer membrane of Gram-negative bacteria. Although

the studies described above have helped us understand the genetics of LPS synthesis (de la Peña-Moctezuma *et al.*, 1999; Kalambaheti *et al.*, 1999), leptospiral LPS structures are still unknown. Thus, Figure 2 reflects a certain amount of artistic license.

A major focus of leptospiral research is identification of OMPs exposed on the leptospiral surface. At least two types of leptospiral OMPs have been characterized. The first type includes transmembrane OMPs, such as porins, that traverse the outer membrane. The second types are outer membrane lipoproteins, which are anchored to the extracellular or periplasmic faces of the outer membrane by fatty acids covalently attached to an amino-terminal cysteine.

The Porin, OmpL1

OmpL1 is the first transmembrane OMP described from a pathogenic spirochete (Haake *et al.*, 1993). OmpL1 was originally isolated in surface immunoprecipitation studies intended to identify proteins exposed on the leptospiral surface (Haake *et al.*, 1991). The N-terminal amino acid sequence obtained from the 33-kDa surface-immunoprecipitated protein was used to design oligonucleotide primers that allowed isolation of the *ompL1* gene (Haake *et al.*, 1993). Examination of the OmpL1 sequence revealed beta-sheet membrane spanning segments typical of Gram-negative OMPs (Haake *et al.*, 1993), a feature consistent with its property of heat-modifiable electrophoretic mobility (Shang *et al.*, 1995). Polyclonal rabbit antiserum directed to a purified, recombinant His6-OmpL1 fusion protein was used to show OmpL1 surface exposure by immunoelectron microscopy (Haake *et al.*, 1993). Subsequent structural studies found that OmpL1 is an integral membrane protein that is present in the native leptospiral membrane as trimers, another feature typical of gram-negative transmembrane OMPs (Shang *et al.*, 1995). Purified, recombinant OmpL1 exhibits porin activity in planar lipid bilayer studies (Shang *et al.*, 1995). We believe that research on OmpL1 may be relevant to transmembrane OMPs of other pathogenic spirochetes.

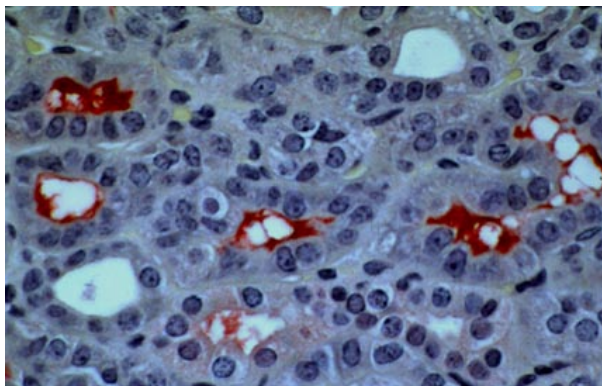


Figure 3. Immunohistochemistry of kidney tissue from a hamster. Tissue from a hamster infected with virulent *Leptospira* was reacted with LipL32 antiserum. Antigen (red) was detected on leptospires adherent to the renal tubular epithelial surface. Photo credit: Prof. Jeanne Barnett, University of Southern Indiana (used with permission).

This area is of great interest because proteins of this type are potential targets of a protective host immune response (Bunikis *et al.*, 1995; Skare *et al.*, 1996).

Leptospiral Lipoproteins

Like other bacterial lipoproteins, leptospiral lipoproteins are likely anchored to membranes by fatty acids that modify the amino-terminal cysteine. We have isolated the genes encoding six different leptospiral lipoproteins, five of which are either exclusively or partly in the outer membrane. We designated the leptospiral lipoproteins based upon their apparent molecular mass, similar to Norris' system of treponemal protein nomenclature (Norris, 1993). For example, our first leptospiral lipoprotein was designated LipL41 because it has an apparent molecular mass of 41-kDa.

LipL41 was initially identified in the same surface-immunoprecipitation studies that isolated OmpL1 (Haake *et al.*, 1991). Subsequent surface-immunoprecipitation experiments confirmed that LipL41 is exposed on the leptospiral surface, while another outer membrane lipoprotein, LipL36, is restricted to the periplasmic leaflet of the outer membrane (Shang *et al.*, 1996). The most abundant proteins in spirochetes are lipoproteins, and *Leptospira* species are no exceptions to this rule. A 32 kDa protein was identified as the major OMP by detergent solubilization of the outer membrane (Zuerner *et al.*, 1991). This major OMP was subsequently shown to be the lipoprotein, LipL32 (Haake *et al.*, 2000).

In each leptospiral lipoprotein, the deduced amino acid sequence reveals a signal peptide followed by a lipoprotein signal peptidase cleavage site. The lipoprotein signal peptidase cleavage sites of LipL41 (LGNC) and LipL36 (LTAC) are consistent with the *E. coli* L-X-Y-C lipobox, while others, such as that of LipL32 (ITAC) are more similar to those of lipoproteins of other spirochetes. Good evidence for relaxing the lipobox requirements in spirochetes exists. In a review of spirochetal lipoproteins (Haake, 2000), 26 spirochetal protein sequences for which there is experimental evidence of lipid modification were compared allowing definition of the spirochetal "lipobox" as:

-Leu (Ala, Ser)₄-Leu (Val, Phe, Ile)₃-Ile (Val, Gly)₂-Ala (Ser, Gly)₁-Cys₊₁-

Several other lines of evidence also support the conclusion that leptospiral lipoproteins are modified by lipid at a N-terminal cysteine residue. Lipid modification seems responsible for the hydrophobicity of these proteins. Native LipL32, LipL36, LipL41, and LipL48 partition into the Triton X-114 detergent phase, while the corresponding His6 fusion proteins partition into the Triton X-114 aqueous phase. Intrinsic labeling of *L. kirschneri* with tritiated palmitate results in selective labeling of LipL32, LipL36, and LipL41. With LipL32, acid treatment resulted in removal of the tritium label, consistent with the hydrolysis of the linkage between palmitate and the amino-terminal cysteine. For LipL36 and LipL41, lipidation is sensitive to globomycin, a selective inhibitor of lipoprotein signal peptidase.

OMPs Expressed During Mammalian Infection

Transmission of leptospirosis is often indirect. Organisms living in the renal tubules of a reservoir host are shed in

the urine and can survive, at least transiently, in the ambient environment before invading a new mammalian host. We believe that the need to cope with these two very different environmental conditions explains why several leptospiral membrane proteins we have studied are differentially expressed. Outer membrane proteins expressed during mammalian infection would be of greater interest as targets for a protective immune response. Although, given the success of OspA immunization in prevention of *B. burgdorferi* infection, it is possible for proteins that are not expressed during mammalian infection to be relevant vaccinogens (Schwan *et al.*, 1995; Sigal *et al.*, 1998; Steere *et al.*, 1998).

Using kidney tissue obtained from hamsters infected with *L. kirschneri*, immunohistochemistry studies have been done to detect the expression and distribution of outer membrane antigens during renal infection (Figure 3) (Barnett *et al.*, 1999; Haake *et al.*, 2000). Hamsters were challenged with host-derived *L. kirschneri* to generate sera that contained antibodies to antigens expressed *in vivo*. Immunoblots using this sera reacted with OmpL1, LipL32, and LipL41, but not LipL36. Although LipL36 is a prominent outer membrane antigen of cultivated *L. kirschneri*, its expression also could not be detected in infected hamster kidney tissue by immunohistochemistry. These data show that expression of this protein is down-regulated *in vivo*. In contrast, LPS, OmpL1, LipL41, and LipL32 (Figure 3) can be shown on organisms colonizing the lumen of proximal convoluted renal tubules at both 10 and 28 days post infection.

Strategies for Recombinant Expression of Leptospiral OMPs

Specialized expression plasmids were developed to simplify expression of leptospiral proteins in *E. coli* as the membrane-associated proteins, OmpL1-M and LipL41-M (the -M designation is used to distinguish these proteins from non-membranous forms). Although OmpL1-M expression is highly toxic in *E. coli*, expression was accomplished using the low-copy-number pMMB66 plasmid. The intact *ompL1* gene, including its signal peptide, was inserted into the pMMB66 plasmid. This construct allowed OmpL1 to be inserted into the *E. coli* outer membrane (Shang *et al.*, 1995). While inclusion of a signal peptide allows membrane association to occur, it also creates difficulties with toxicity and plasmid stability. This would have been expected to be a problem considering that expression using pMMB66 is under control of a leaky *tac* promoter. However, the pMMB66-OmpL1 plasmid had undetectable background expression without inducer (Haake *et al.*, 1999). This observation was explained by finding a spontaneous mutation within the ribosome-binding site of the pMMB66-OmpL1 plasmid. We believe that this fortuitous arrangement eliminates toxic background expression while still allowing efficient expression after IPTG induction. This modification of a *tac* promoter-containing expression plasmid may be superior to other approaches for expression of bacterial OMPs that are highly toxic to *E. coli*.

Membrane association of LipL41 is dependent on modification of the N-terminal cysteine by fatty acids. However, expression of LipL41 in *E. coli* using plasmids containing the native lipL41 gene resulted in poor yields

and incomplete processing by *E. coli* lipoprotein signal peptidase. LipL41 appeared to have a typical lipoprotein signal peptidase cleavage site, with a leucine at the -3 position relative to cysteine. Nevertheless, we wondered whether the amino acids between the leucine and the cysteine were responsible for the poor processing of LipL41 in *E. coli*. The arginine at the -1 position was of particular concern, since amino acids alanine or glycine with small neutral side-chains are often found at this position in most bacterial lipoproteins. We used a PCR mutagenesis strategy to alter the pET-15b-LipL41 plasmid sequence. The LipL41 lipoprotein signal peptidase cleavage site was changed from LGNC to LAGC, *i.e.*, mimicking that of the abundant murein lipoprotein. Expression of LipL41 using the modified pET-15b-LipL41 plasmid resulted in high-level expression of lipidated recombinant LipL41 (Haake *et al.*, 1999). Based on studies with OspA of *Borrelia burgdorferi* (Erdile *et al.*, 1993), we felt that a lipidated form of LipL41 would serve as a better immunogen than an unlipidated form.

Immunoprotection Studies in Hamsters

We have examined the immunoprotective capacity of the leptospiral porin, OmpL1, and the leptospiral outer membrane lipoprotein, LipL41, in the Golden Syrian hamster model of leptospirosis. Using the expression strategies described in the previous section, these two leptospiral proteins were expressed in *E. coli* as membrane proteins, OmpL1-M and LipL41-M. Active immunization of hamsters with *E. coli* membrane fractions containing a combination of OmpL1-M and LipL41-M provided significant protection against homologous challenge with *L. kirschneri* serovar grippotyphosa (Haake *et al.*, 1999). Twenty eight days after intraperitoneal inoculation, survival in animals vaccinated with both proteins was 71% (95% CI, 53% to 89%), compared with only 25% (95% CI, 8% to 42%) in the control group ($P < 0.001$). No evidence of infection was found in the vaccinated survivors using serological, histological, and microbiological assays. Interestingly, the protective effects of immunization with OmpL1-M and LipL41-M were synergistic, since we did not observe significant levels of protection in animals immunized with the membrane-associated forms of either OmpL1 or LipL41 alone. In contrast, hamsters immunized with detergent-solubilized His6-OmpL1 and His6-LipL41 fusion proteins, either alone or in combination, were not protected. These data show that the manner in which OmpL1 and LipL41 are formulated is an important determinant of immunoprotection.

Future Directions

Analysis of closely related strains of *L. borgpetersenii* serovar hardjo and *L. interrogans* serovars icterohaemorrhagiae and pomona suggests both IS1500 and IS1533 may be transpositionally active. These data combined with the recent success constructing a shuttle vector (Saint Girons *et al.*, 2000) suggest that these IS elements may be useful in genetic manipulation of *Leptospira* spp. Characterization of transcriptional regulation is helping to allow more detailed analysis of these bacteria, and these techniques should be applicable to studying the expression of genes in different environments.

Therefore, understanding the extracellular signals that affect membrane protein expression and other genes associated with physiological changes as the bacteria enter new environments should be possible. Analysis of OMP genes provides insight to *Leptospira* cell structure, and will allow for the development of more effective vaccines. The prospect of soon possessing the genome sequence should also open these bacteria to more careful scrutiny and should be useful in identifying additional targets for vaccine and drug development to aid in controlling leptospirosis.

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